BD SMART™ RACE cDNA Amplification Kit User Manual

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I. Introduction & Protocol Overview

The **BD SMART™ RACE cDNA Amplification Kit** provides a novel method for performing both 5'- and 3'-rapid amplification of cDNA ends (RACE). This kit integrates our BD Marathon™ cDNA Amplification Kit (Chenchik *et al.*, 1995; 1996) with our BD SMART (Switching Mechanism At 5' end of RNA Transcript) cDNA synthesis technology. This powerful combination allows you to isolate the complete 5' sequence of your target transcript more consistently than ever before. Furthermore, BD SMART technology eliminates the need for problematic adaptor ligation and lets you use first-strand cDNA directly in RACE PCR, a benefit that makes RACE far less complex and much faster (Chenchik *et al.*, 1998). The BD SMART RACE Kit also includes recent advances in PCR technology that both increase the sensitivity and reduce the background of the RACE reactions. As a result you can use either poly A^+ or total RNA as starting material for constructing full-length cDNAs of even very rare transcripts.

BD SMART technology provides a mechanism for generating full-length cDNAs in reverse transcription reactions (Zhu *et al.*, 2001). This is made possible by the joint action of the BD SMART II™ A Oligonucleotide and the BD PowerScript™ Reverse Transcriptase (RT). BD PowerScript RT is a variant of MMLV RT that, upon reaching the end of an RNA template, exhibits terminal transferase activity, adding 3–5 residues (predominantly dC) to the 3' end of the first-strand cDNA (Figure 1). The BD SMART oligo contains a terminal stretch of G residues that anneal to the dC-rich cDNA tail and serves as an extended template for RT. BD PowerScript RT switches templates from the mRNA molecule to the BD SMART oligo, generating a complete cDNA copy of the original RNA with the additional BD SMART sequence at the end. Since the dC-tailing activity of RT is most efficient if the enzyme has reached the end of the RNA template, the BD SMART sequence is typically added only to complete first-strand cDNAs. This process guarantees that the use of high quality RNA will result in the formation of a set of cDNAs that have a maximum amount of 5' sequence (Table I).

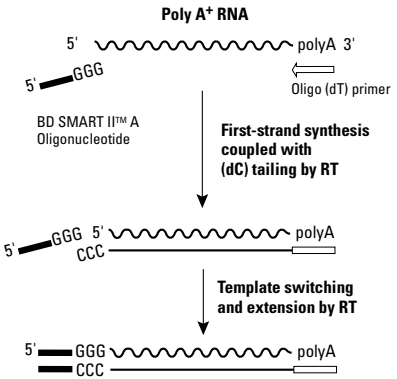

Figure 1. Mechanism of BD SMART™ cDNA synthesis. First-strand synthesis is primed using a modified oligo (dT) primer. After reverse transcriptase reaches the end of the mRNA template, it adds several dC residues. The BD SMART II A Oligonucleotide anneals to the tail of the cDNA and serves as an extended template for BD PowerScript RT.

I. Introduction & Protocol Overview *continued*

Following reverse transcription, the first-strand cDNA is used directly in 5'- and 3'-RACE PCR reactions, without the need for tedious second-strand synthesis and adaptor ligation. The incorporation of BD SMART technology also permits the use of "universal priming" in the RACE PCR amplification. This method, along with the techniques of suppression PCR and step-out PCR ensure high specificity in amplifying your target cDNA. These methods are described in detail below and in Appendix C.

The only requirement for BD SMART RACE cDNA amplification is that you know at least 23–28 nucleotides (nt) of sequence information in order to design gene-specific primers (GSPs) for the 5'- and 3'-RACE reactions. (Additional sequence information will facilitate analysis of your RACE products.) This limited requirement makes BD SMART RACE ideal for characterizing genes identified through diverse methods including cDNA subtraction, differential display, RNA fingerprinting, ESTs, library screening, and more.

BD SMART RACE cDNA amplification is a flexible tool—many researchers use this kit in place of conventional kits to amplify just the 5' or 3' end of a particular cDNA. Others perform *both* 5'- and 3'-RACE, and many then go on to clone full-length cDNAs using one of the two methods described in the latter part of this protocol. In many cases, researchers obtain full-length cDNAs without ever constructing or screening a cDNA library.

TABLE I: ADDITIONAL 5'-RACE SEQUENCE OBTAINED WITH BD SMART TECHNOLOGY

Human gene	Size of mRNA (kb)	Additional sequence (bp)*	Matches genomic sequence	Includes transcription start site
Transferrin receptor	5.0	+25	yes	yes
Smooth muscle g-actin	1.28	+31	yes	yes
Vascular smooth muscle α-actin	1.33	+17	yes	yes
Cytoskeletal γ-actin	1.9	+1	yes	yes
23 kDa HBP	0.67	+9	n/a	yes
p53	2.6	+4	yes	yes
Interferon-γ receptor	2.06	+14	yes	yes
14-3-3 protein	1.03	+1	n/a	n/a
Interferon-α receptor	2.75	+17	yes	yes

n/a = not available
* Compared to GenBank cDNA sequence

I. Introduction & Protocol Overview *continued*

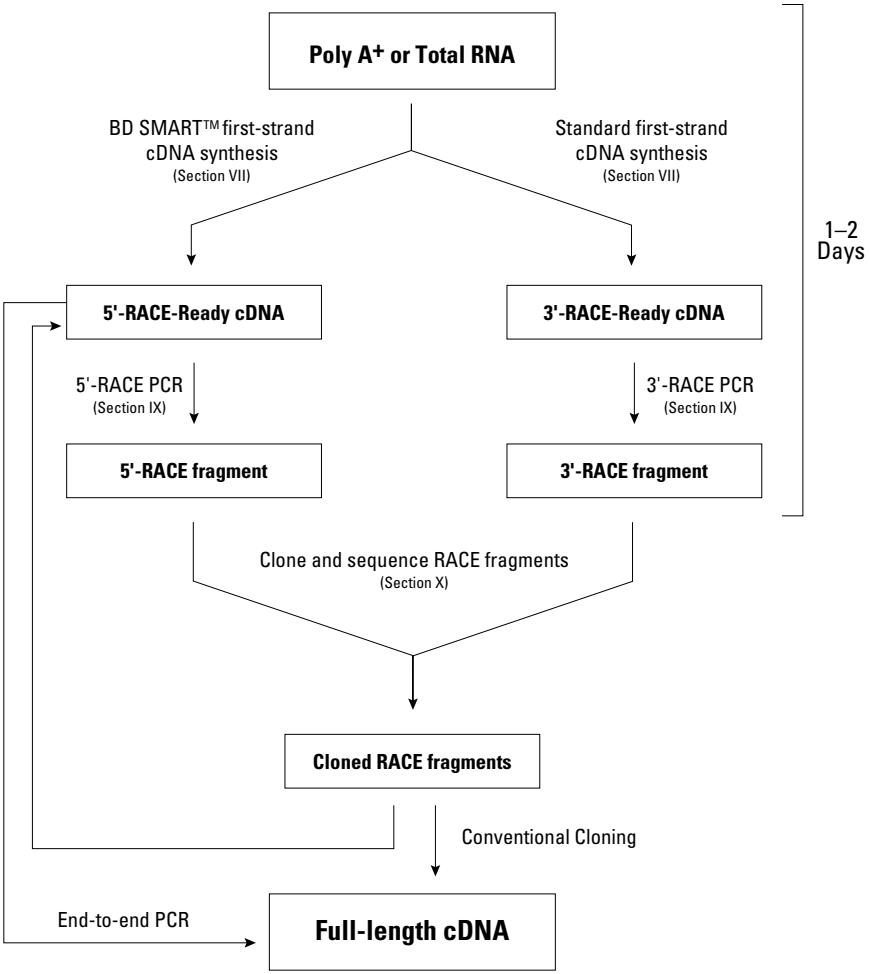

Figure 2. Overview of the BD SMART™ RACE procedure. Detailed flow charts of the BD SMART RACE mechanisms can be found in Appendices A & B. Note that with the cloned RACE fragments you can use a restriction site in an overlapping region to construct a full-length cDNA by subcloning. Alternatively, you can sequence the 5' end of the 5' product and the 3' end of the 3' product to obtain the sequences of the extreme ends of the transcript. Using this information, you can design 5' and 3' gene-specific primers to use in LD PCR with the 5'-RACE-Ready cDNA as template to generate the full-length cDNA.

I. Introduction & Protocol Overview *continued*

Overview of the BD SMART RACE cDNA amplification protocol

An overview of the BD SMART RACE cDNA amplification is presented in Figure 2. Detailed mechanisms of the RACE reactions are provided in Appendices A & B.

- **Primer Design** (Section V)

  You must design gene-specific primers for the 5'- and/or 3'-RACE reactions (GSP1 and GSP2, respectively). As described, nested primers (NGSP1 and NGSP2) will facilitate analysis of your RACE products. They can also be used for nested RACE PCR if necessary. Primer design is discussed in detail in Section V; Figure 3 shows the relationship of primers and template used in BD SMART RACE reactions.

- **First-strand cDNA synthesis** (Section VII)

  Since the 5' elongation benefits of BD SMART technology are only relevant for 5'-RACE, the BD SMART RACE Kit includes a protocol for the synthesis of two separate cDNA populations: 5'-RACE-Ready cDNA and 3'-RACE-Ready cDNA. The cDNA for 5'-RACE is synthesized using a modified lock-docking oligo(dT) primer and the BD SMART II A oligo as described above. The modified oligo(dT) primer, termed the 5'-RACE CDS Primer (5'-CDS), has two degenerate nucleotide positions at the 3' end. These nucleotides position the primer at the start of the poly A^+ tail and thus eliminate the 3' heterogeneity inherent with conventional oligo(dT) priming (Borson *et al.*, 1994).

  The 3'-RACE cDNA is synthesized using a traditional reverse transcription procedure, but with a special oligo(dT) primer. This 3'-RACE CDS Primer A (3'-CDS) primer includes the lock-docking nucleotide positions as in the 5'-CDS primer and also has a portion of the BD SMART sequence at its 5' end. By incorporating the BD SMART sequence into both the 5'- and 3'-RACE-Ready cDNA populations, you can prime *both* RACE PCR reactions using the Universal Primer A Mix (UPM), which recognizes the BD SMART sequence, in conjunction with distinct gene-specific primers.

- **Positive Control RACE Experiment** (Section VIII)

  Prior to performing RACE with your template, we strongly recommend that you perform the positive control RACE experiment using the Control Human Placental Total RNA provided in the kit.

- **RACE PCR Reactions** (Section IX)

  After you generate RACE-Ready cDNAs, you will have enough material to perform 5'- and 3'-RACE with many different genes, simply by using different gene-specific primers. All PCR reactions in the BD SMART RACE protocol are optimized for use with the BD Advantage™ 2 Polymerase Mix. The Polymerase Mix is comprised of BD TITANIUM™ *Taq* DNA Polymerase—a

I. Introduction & Protocol Overview *continued*

nuclease-deficient N-terminal deletion of *Taq* DNA polymerase plus BD TaqStart™ Antibody to provide automatic hot-start PCR (Kellogg *et al.*, 1994)—and a minor amount of a proofreading polymerase. BD Advantage 2 technology enables you to perform long distance PCR (LD PCR) reactions with confidence that your products will have high fidelity to the original sequences (Barnes, 1994; Cheng *et al.*, 1994). As a result, you will be able to amplify longer templates than were possible in traditional RACE procedures.

- **Characterization of RACE Products** (Section X)

  Before constructing your full-length cDNA, we strongly recommend that you confirm amplification of the desired target. You can characterize your RACE products by one or more of the following: (1) comparing PCR products obtained using GSP1 and UPM to products generated with NGSP1 and UPM; (2) probing a Southern blot of your PCR products with an internal gene-specific probe (e.g., labeled NGSP1); and (3) cloning and sequencing your RACE products. In general, we recommend that you obtain at least some sequence information.

  Careful characterization of your RACE products at this point can prevent confusion and wasted effort in your subsequent experiments, even when both RACE reactions produce single major products. This analysis is especially important if you have multiple RACE products or suspect that you are working with a member of a multigene family.

Note on "full-length" cDNAs: No method of cDNA synthesis can guarantee a full-length cDNA, particularly at the 5' end. Determining the true 5' end requires some combination of RNase protection assays, primer extension assays, and cDNA or genomic sequence information. Many BD SMART RACE cDNAs include the complete 5' end of the cDNA; however, severe secondary structure may block the action of RT and/or *Taq* DNA polymerase in some instances. In our experience, BD SMART RACE products and full-length cDNAs compare favorably in this regard with cDNAs obtained by conventional RACE or from libraries. To obtain the maximum possible amount of 5' sequence, we recommend that you sequence the 5' end of 5–10 separate clones of the 5'-RACE product.

II. List of Components

Store Control Human PlacentalTotal RNA and BD SMART II A Oligonucleotide at –70°C.

Store NucleoTrap Gel Extraction Kit at room temperature.

Store all other reagents at –20°C.

Purchase of the BD SMART RACE cDNA Amplification Kit includes a free sample of BD PowerScript Reverse Transcriptase and a free trial-size BD Advantage 2 PCR Kit (Cat. No. 639207). Together, these products provide sufficient reagents for 7 cDNA synthesis reactions and 30 PCR reactions.

First-strand cDNA Synthesis

- 7 µl **BD SMART II™ A Oligonucleotide** (12 µM)
  5'–AAGCAGTGGTATCAACGCAGAGTACGCGGG–3'
- 7 µl **3'-RACE CDS Primer A** (3'-CDS; 12 µM)
  5'–AAGCAGTGGTATCAACGCAGAGTAC$(T)_{30}$V N–3'
  (N = A, C, G, or T; V = A, G, or C)
- 7 µl **5'-RACE CDS Primer** (5'-CDS; 12 µM)
  5'–$(T)_{25}$V N–3'
  (N = A, C, G, or T; V = A, G, or C)
- 7 µl **BD PowerScript™ Reverse Transcriptase**
- 200 µl **5X First-Strand Buffer**
  250 mM Tris-HCl (pH 8.3)
  375 mM KCl
  30 mM $MgCl_2$
- 200 µl **Dithiothreitol** (DTT; 20 mM)
- 1 ml **Deionized H_2O**

5'- & 3'-RACE PCR

- 400 µl **10X Universal Primer A Mix** (UPM)
  Long (0.4 µM):
  5'–CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT–3'
  Short (2 µM):
  5'–CTAATACGACTCACTATAGGGC–3'
- 50 µl **Nested Universal Primer A** (NUP; 10 µM)
  5'–AAGCAGTGGTATCAACGCAGAGT–3'

Control Reagents

- 5 µl **Control Human Placental Total RNA** (1 µg/µl)
- 25 µl **Control 5'-RACE TFR Primer** (10 µM)
- 25 µl **Control 3'-RACE TFR Primer** (10 µM)

II. List of Components *continued*

General Reagents

- 70 µl **dNTP Mix** (dATP, dCTP, dGTP, and dTTP, each at 10 mM)
- 2 X 1 ml **Tricine-EDTA Buffer**
  10 mM Tricine-KOH (pH 8.5)
  1.0 mM EDTA

NucleoTrap® Gel Extraction Kit (Cat. No. 636053 or K3070-y)

- 100 µl NucleoTrap Suspension
- 3 ml NT1 Buffer
- 10 ml NT2 Buffer
- 2 ml NT3 Buffer
- User Manual (PT3169-1)

Free trial-size BD Advantage™ 2 PCR Kit (Cat. No. 639207 or K1910-y)

The BD Advantage 2 kit provides sufficient reagents for 30 PCR reactions. The following components are included:

- 30 µl 50X BD Advantage 2 Polymerase Mix
- 200 µl 10X BD Advantage 2 PCR Buffer
- 50 µl 50X dNTP Mix (10 mM each)
- 30 µl Control DNA Template (100 ng/µl)
- 30 µl Control Primer Mix (10 µM each)
- 1 ml PCR-Grade Water
- User Manual (PT3281-1)

III. Additional Materials Required

The following reagents are required but not supplied:

- **0.5-ml PCR reaction tubes**. We recommend PerkinElmer GeneAmp 0.5-ml reaction tubes (Cat. No. N801-0737 or N801-0180).
- **Mineral oil** (e.g., Sigma Cat. No. M-3516)

IV. General Considerations for BD SMART RACE

PLEASE READ ENTIRE PROTOCOL BEFORE STARTING

- The cycling parameters throughout this protocol were optimized with an authorized hot-lid thermal cycler, the BD Advantage 2 Polymerase Mix, and the reagents and TFR controls provided in the BD SMART RACE Kit. The optimal cycling parameters may vary with different polymerase mixes, templates, gene-specific primers, and thermal cyclers. Prior to performing 5'- and 3'-RACE with your experimental sample, you should perform the positive control PCR experiment (Section VIII). These reactions, which use cDNA generated from the Control Human Placental Total RNA and the Control 5'- and 3'- RACE TFR Primers, will help determine if you need to alter the PCR program for your thermal cycler.

  Please note that the efficiency of RACE PCR depends on the abundance of the mRNA of interest in your RNA sample. Additionally, different primers will have different optimal annealing/extension temperatures. Refer to Section XI for suggestions on optimizing PCR conditions.
- **You must use some form of hot start in the 5'-RACE and 3'-RACE PCR reactions.** The following protocols were optimized using the BD Advantage 2 Polymerase Mix which contains BD TaqStart Antibody for automatic hot start PCR (Kellogg *et al.,* 1994). Hot start can also be performed using wax beads (Chou *et al.*, 1992) or manually (D'Aquila *et al.*, 1991).
- We recommend the Tricine-EDTA Buffer provided in the kit for resuspending and diluting your DNA samples throughout this protocol. Tricine buffers maintain their pH at high temperature better than Tris-based buffers. Tris-based buffers can lead to low pH conditions that degrade DNA.
- Wear gloves throughout to protect your RNA samples from nucleases.
- Resuspend pellets and mix reactions by gently pipetting the solution up and down or by tapping the bottom of the tube. Then spin the tube briefly to bring all contents to the bottom.
- Perform all reactions on ice unless otherwise indicated.
- Add enzymes to reaction mixtures last.
- Use the recommended amounts of enzyme. These amounts have been carefully optimized for the BD SMART RACE amplification protocol and reagents.
- Ethidium bromide is a carcinogen. Use appropriate precautions in handling and disposing of this reagent. For more information, see *Molecular Cloning: A Laboratory Manua*l by Sambrook *et al.* (1989).

V. Primer Design

A. Primer Sequence

Gene-Specific Primers (GSPs) should be:

- 23–28 nt
- 50–70% GC
- T_m ≥65°C; best results are obtained if T_m >70°C (enables the use of touchdown PCR)

The relationship of the primers used in the BD SMART RACE reactions to the template and resulting RACE products is shown in detail in Figure 3. For the complete BD SMART RACE protocol, you will need at least two GSPs: an antisense primer for the 5'-RACE PCR and a sense primer for the 3'-RACE PCR. If you are doing only 5'- or 3'-RACE, you will only need one GSP. All primers should be 23–28 nt long; there is generally no advantage to using primers longer than 30 nt. The primers shown in Figure 3 will create overlapping 5'- and 3'-RACE products. If a suitable restriction site is located in the region of overlap, the fragments can subsequently be joined by restriction digestion and ligation to create the full-length cDNA. By designing primers that give a 100–200-bp overlap in the RACE products, you will also be able to use the primers together as a positive control for the PCR reactions. However, it is not absolutely necessary to use primers that give overlapping fragments. In the case of large and/or rare cDNAs, it may be better to use primers that are closer to the ends of the cDNA and therefore do not create overlapping fragments. Additionally, the primers themselves can overlap (i.e., be complementary).

GSPs should have a GC content of 50–70% and a T_m of at least 65°C; whenever possible the T_m should be greater than 70°C, as determined by nearest neighbor analysis (Freier *et al.*, 1986; we use the Primer Premier software to calculate T_m's). In our experience, longer primers with annealing temperatures above 70°C give more robust amplification in RACE, particularly from difficult samples. T_m's over 70°C allow you to use "touchdown PCR" (Section C below). Additionally, designing GSP1 and GSP2 so that they have similar T_m's will facilitate their use in the BD SMART RACE protocol. T_m's of GSP1 and GSP2 can be calculated or determined experimentally by performing PCR at different temperatures. Avoid using self-complementary primer sequences which can fold back and form intramolecular hydrogen bonds. Similarly, avoid primers that have complementarity to the primers in the Universal Primer Mix, particularly in their 3' ends. (See Section II for UPM primer sequences.)

Note: Do not incorporate restriction sites into the 5' ends of the 5' and 3' GSPs. In our experience, these extra sequences can lead to increased background.

V. Primer Design *continued*

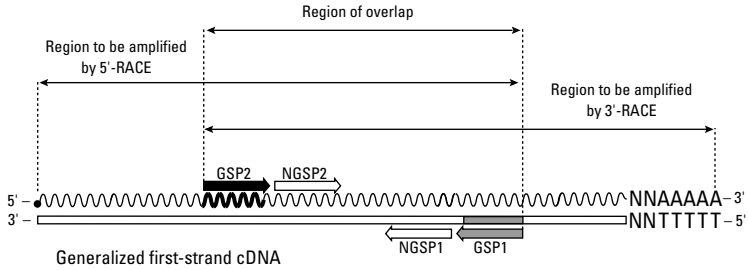

Figure 3. The relationship of gene-specific primers to the cDNA template. This diagram shows a generalized first-strand cDNA template. This RNA/DNA hybrid does not precisely represent either the 5'-RACE-Ready or 3'-RACE-Ready cDNAs. For a detailed look at those structures, see Appendices A & B. Note that the gene-specific primers designed here produce overlapping RACE products. This overlap permits the use of the primers together in a control PCR reaction. Additionally, if a suitable restriction site is located within this region, it will be possible to construct the full-length cDNA by subcloning.

B. Location of Primer Sequences within Gene

We have had good success using the BD SMART RACE Kit to amplify 5' and 3' cDNA fragments that extend up to 6.5 kb from the GSP sites. Nevertheless, for optimum results we recommend choosing your primers so that the 5'- and 3'-RACE products will be 2 kb or less.

C. Touchdown PCR

We have found that touchdown PCR (Don *et al.*, 1991; Roux, 1995) significantly improves the specificity of BD SMART RACE amplification. Touchdown PCR uses an annealing temperature during the initial PCR cycles that is *higher* than the T_m of the Universal Primer. If the T_m of your GSP is >70°C, *only* gene-specific synthesis occurs during these cycles, allowing a critical amount of gene-specific product to accumulate. The annealing temperature is then reduced to a level compatible with the UPM, permitting efficient, exponential amplification of the gene-specific template. (See Appendices A–C for more details.)

As noted above, we recommend using primers with T_m's >70°C to allow you to use the touchdown cycling programs in the protocol. (Non-touchdown cycling programs are also included for use with primers with T_m's <70°C.)

D. Nested Primers

We recommend that you do *not* use nested PCR in your initial experiments. The UPM Primer and a GSP will usually generate a good RACE product with a low level of nonspecific background. However, Southern blotting with nested GSPs (NGSP1 and NGSP2) as probes is useful for characterizing your RACE products. Furthermore, nested PCR may be necessary in some

V. Primer Design *continued*

cases where the level of background or nonspecific amplification in the 5'- or 3'-RACE reaction is too high with a single GSP. In nested PCR, a primary amplification is performed with the outer primers and, if a smear is produced, an aliquot of the primary PCR product is reamplified using the inner primers. The BD SMART RACE protocols include optional steps indicating where nested primers can be used. The Nested Universal Primer A (provided with the kit) can be used for both 5'- and 3'-RACE.

Nested gene specific primers should be designed according to the guidelines discussed above. If possible, nested primers should not overlap with the outer gene-specific primers; if they must overlap due to limited sequence information, the 3' end of the inner primer should have as much unique sequence as possible.

VI. Preparation & Handling of Total and Poly A^+ RNA

A. General Precautions

The integrity and purity of your total or poly A^+ RNA starting material is an important element in high-quality cDNA synthesis. The following precautions will help you avoid contamination and degradation of your RNA:

- Wear gloves.
- Use freshly deionized (e.g., MilliQ-grade) H_2O directly, without treatment with DEPC (diethyl pyrocarbonate).
- Rinse all glassware with 0.5 N NaOH, followed by deionized H_2O. Then bake the glassware at 160–180°C for 4–9 hr.
- Use only single-use plastic pipettes and pipette tips.

B. RNA Isolation

BD Biosciences Clontech offers several kits for the purification of total RNA such as the NucleoBond® RNA/DNA Mini Kit (Cat. No. 635945). Many procedures are available for the isolation of poly A^+ RNA (Farrell, 1993; Sambrook *et al.,* 1989).

C. RNA Analysis

We recommend that you examine your RNA by electrophoresing a sample on a denaturing formaldehyde agarose/EtBr gel. Mammalian total RNA typically exhibits two bright bands at 4.5 and 1.9 kb; these bands correspond to 28S and 18S ribosomal RNA, respectively. The ratio of intensities of these bands should be about 1–2:1. Poly A^+ RNA samples from mammalian cells should produce smears from 0.5–12 kb with much weaker ribosomal RNA bands. Size distribution may be smaller with nonmammalian tissue sources.

VII. First-Strand cDNA Synthesis

The two 10-µl reactions described below convert 50 ng–1 µg of total or poly A^+ RNA into RACE-Ready first-strand cDNA.

We recommend that you use poly A^+ RNA whenever possible. However, if you have less than 50 µg of total RNA we do not recommend purification of poly A^+ RNA because the final yield will be too small to effectively analyze the RNA quantity and quality. For optimal results, use 1 µg of poly A^+ RNA or 1 µg of total RNA in the reactions below.

We strongly recommend that you perform a positive control cDNA synthesis using the included Human Placental Total RNA in addition to your experimental reactions. This cDNA will be used in the positive control RACE reactions in Section VIII.

1. Combine the following in separate 0.5-ml microcentrifuge tubes:

For preparation of 5'-RACE-Ready cDNA		For preparation of 3'-RACE-Ready cDNA	
1–3 µl	RNA sample*	1–3 µl	RNA sample*
1 µl	5'-CDS primer	1 µl	3'-CDS primer A
1 µl	BD SMART II A oligo		

* For the control synthesis, use 1 µl of Control Human Placental Total RNA (1 µg/µl).

2. Add sterile H_2O to a final volume of 5 µl for each reaction.
3. Mix contents and spin the tubes briefly in a microcentrifuge.
4. Incubate the tubes at 70°C for 2 min.
5. Cool the tubes on ice for 2 min.
6. Spin the tubes briefly to collect the contents at the bottom.
7. Add the following to each reaction tube (already containing 5 µl):

2 µl	5X First-Strand Buffer
1 µl	DTT (20 mM)
1 µl	dNTP Mix (10 mM)
1 µl	BD PowerScript Reverse Transcriptase
10 µl	**Total volume**

8. Mix the contents of the tubes by gently pipetting.
9. Spin the tubes briefly to collect the contents at the bottom.
10. Incubate the tubes at 42°C for 1.5 hr in an air incubator or a hot-lid thermal cycler.

    Note: Using a water bath or thermal cycler for this incubation may reduce the volume of the reaction mixture (due to evaporation), and therefore reduce the efficiency of first-strand synthesis.

VII. First-Strand cDNA Synthesis *continued*

11. Dilute the first-strand reaction product with Tricine-EDTA Buffer:
    - Add 20 µl if you started with <200 ng of total RNA.
    - Add 100 µl if you started with >200 ng of total RNA.
    - Add 250 µl if you started with poly A^+ RNA.
12. Heat tubes at 72°C for 7 min.
13. Samples can be stored at –20°C for up to three months.

At this point, you have 3'- and 5'-RACE-Ready cDNA samples. The RACE reactions in Section IX use only a fraction of this material for each RNA of interest. There is sufficient single-stranded cDNA for PCR amplification of multiple genes.

If you intend to use LD PCR to construct your full-length cDNA after completing 5'- and 3'-RACE, be sure to set aside an aliquot of the 5'-RACE-Ready cDNA to use as a template in the PCR reaction.

VIII. Positive Control PCR Experiment

Prior to performing 5'- and 3'-RACE reactions with your cDNA, we *strongly* recommend that you perform the following positive control RACE PCR experiment using the RACE-Ready cDNAs generated from the Control Human Placental Total RNA. These reactions will amplify the ends of the transferrin receptor (TFR) cDNA. This procedure can save you considerable time by ensuring that the BD SMART RACE protocol works with your thermal cycler. If problems arise later in the protocol, the results of this experiment will help you determine immediately if the problem is with your RACE PCR (e.g., different thermal cycler) or with your cDNA.

We recommend that you first perform BD SMART RACE PCR reactions using the BD Advantage 2 Polymerase Mix supplied. If your cDNA of interest has high GC content you can use the BD Advantage™ GC 2 Polymerase Mix (Cat. No. 639114) or PCR Kit (Cat. Nos. 639119 & 639120) for subsequent analysis. For applications in which the highest fidelity product is desired, the BD Advantage™ HF 2 PCR Kit (Cat. Nos. 639123 & 639124) can amplify templates up to 3.5 kb. For more information, see Section XI (Troubleshooting Guide).

1. Prepare enough Master Mix for all PCR reactions and 1 extra reaction to ensure sufficient volume. For each 50-µl PCR reaction, mix the following reagents:

34.5 µl	PCR-Grade Water
5 µl	10X BD Advantage 2 PCR Buffer
1 µl	dNTP Mix (10 mM; in BD SMART RACE or BD Advantage 2 PCR Kit)
1 µl	50X BD Advantage 2 Polymerase Mix
41.5 µl	**Total volume**

2. Mix well by vortexing (without introducing bubbles), then briefly spin the tube in a microcentrifuge.
3. Prepare PCR reactions as shown in Table II. Add the components to 0.5-ml PCR tubes in the order shown and mix gently.

VIII. Positive Control PCR Experiment *continued*

TABLE II: SETTING UP THE POSITIVE CONTROL RACE EXPERIMENT

Component	Tube No.: 1 Description: 5'-RACE Control	2 3'-RACE Control	3 Internal Control (5' cDNA)	4 Internal Control (3' cDNA)
Control 5'-RACE-Ready cDNA	2.5 µl	—	2.5 µl	—
Control 3'-RACE-Ready cDNA	—	2.5 µl	—	2.5 µl
5'-RACE TFR Primer (10 µM)	1 µl	—	1 µl	1 µl
3'-RACE TFR Primer (10 µM)	—	1 µl	1 µl	1 µl
UPM (10X)	5 µl	5 µl	—	-—
H_2O	—	—	4 µl	4 µl
Master Mix	41.5 µl	41.5 µl	41.5 µl	41.5 µl
Final volume	50 µl	50 µl	50 µl	50 µl
Expected product size	2.6 kb	2.9 kb	0.3 kb	0.3 kb

4. Overlay the contents of each tube with 2 drops of mineral oil and place caps firmly on each tube.

   Note: Mineral oil is not necessary if you are using a hot-lid thermal cycler.

5. Commence thermal cycling using the following program for touchdown PCR.

   - 5 cycles:

     94°C 30 sec
     72°C 3 min

   - 5 cycles:

     94°C 30 sec
     70°C 30 sec
     72°C 3 min

   - 27 cycles:

     94°C 30 sec
     68°C 30 sec
     72°C 3 min

6. Analyze 5 µl of each sample on a 1.2 % agarose/EtBr gel. Store the remaining 45 µl of each reaction at –20°C until you are sure the control experiment has worked.

VIII. Positive Control PCR Experiment *continued*

Expected results (see lanes 2 and 5 of the gels in Figure 4): The 5'-RACE control reaction should produce a 2.6-kb band. The 3'-RACE control reaction should produce a 2.9-kb band. If you do not observe these bands, return the tube(s) to your PCR machine and try cycling the remaining portion of the reaction for 5 additional cycles. If you still do not see the desired product, consult Section XI for troubleshooting. Before you attempt 5'- and 3'-RACE with your primers and experimental cDNA, we recommend that the positive control reactions produce single strong bands of the correct size in 42 or fewer total cycles (5 cycles annealing at 72°C + 5 cycles at 70°C + 32 cycles at 68°C).

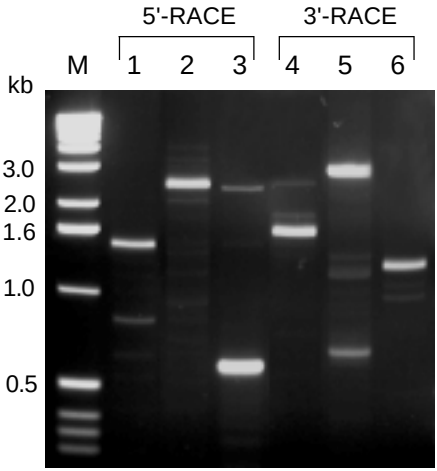

Figure 4. 5'- and 3'-RACE sample results. At BD Biosciences Clontech, we have used the BD SMART RACE Kit to amplify 5'- and 3'-RACE fragments of many different genes starting with poly A^+ and total RNA. This gel shows several representative 5'- and 3'-RACE amplifications starting with total RNA. Lanes 1 & 4: interferon-γ receptor. Lanes 2 & 5: transferrin receptor. Lanes 3 & 6: HPRT. The control PCR reactions described for transferrin receptor amplification should produce the RACE products in lanes 2 & 5. The 5' product will be 2.6 kb; the 3' product will be 2.9 kb. As seen here, a minor 0.6-kb product will occasionally be generated in transferrin receptor 3'-RACE.

IX. Rapid Amplification of cDNA Ends (RACE)

This procedure describes the 5'-RACE and 3'-RACE PCR reactions that generate the 5' and 3' cDNA fragments. We recommend that you also perform positive control 5'- and 3'-RACE using the TFR primers, UPM, and control RACE-Ready cDNAs as described in Section VIII. Although the Nested Universal Primer A (NUP) is provided, nested PCR is generally not necessary in BD SMART RACE reactions.

Please note that all RACE PCR reactions have been optimized for use with the BD Advantage 2 Polymerase Mix.

1. Prepare enough PCR Master Mix for all PCR reactions and one extra reaction to ensure sufficient volume. The same Master Mix can be used for both 5'- and 3'-RACE reactions. For each 50-µl PCR reaction, mix the following reagents:

34.5 µl	PCR-Grade Water
5 µl	10X BD Advantage 2 PCR Buffer
1 µl	dNTP Mix (10 mM)
1 µl	50X BD Advantage 2 Polymerase Mix
41.5 µl	**Total volume**

2. Mix well by vortexing (without introducing bubbles), then briefly spin the tube in a microcentrifuge.
3. **For 5'-RACE:** prepare PCR reactions as shown in Table III.

   For 3'-RACE: prepare PCR reactions as shown in Table IV.

   Add the components to 0.5-ml PCR tubes in the order shown and mix gently.

TABLE III: SETTING UP 5'-RACE PCR REACTIONS

Component	1 5'-RACE Sample	2 5'-TFR* (+ Control)	3 GSP 1 + 2† (+ Control)	4 UPM only (– Control)	5 GSP1 only (– Control)
5'-RACE-Ready cDNA (experimental)	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl
UPM (10X)	5 µl	5 µl	—	5 µl	—
GSP1 (10 µM)	1 µl	—	1 µl	—	1 µl
GSP2 (10 µM)	—	—	1 µl	—	—
Control 5'-RACE TFR Primer (10 µM)	—	1 µl	—	—	—
H_2O	—	—	4 µl	1 µl	5 µl
Master Mix	41.5 µl	41.5 µl	41.5 µl	41.5 µl	41.5 µl
Final volume	50 µl	50 µl	50 µl	50 µl	50 µl

* Skip this reaction if your RNA is nonhuman.
† Skip this reaction if your GSPs *will not* create overlapping RACE fragments.
For detailed descriptions of the control reactions, see Section XI.

IX. Rapid Amplification of cDNA Ends (RACE) *continued*

TABLE IV: SETTING UP 3'-RACE PCR REACTIONS

Component	1 3'-RACE Sample	2 3'-TFR* (+ Control)	3 GSP 1 + 2† (+ Control)	4 UPM only (– Control)	5 GSP2 only (– Control)
3'-RACE-Ready cDNA (experimental)	2.5 µl	2.5 µl	2.5 µl	2.5 µl	2.5 µl
UPM (10X)	5 µl	5 µl	—	5 µl	—
GSP1 (10 µM)	—	—	1 µl	—	—
GSP2 (10 µM)	1 µl	—	1 µl	—	1 µl
Control 3'-RACE TFR Primer (10 µM)	—	1 µl	—	—	—
H_2O	—	—	4 µl	1 µl	5 µl
Master Mix	41.5 µl	41.5 µl	41.5 µl	41.5 µl	41.5 µl
Final volume	50 µl	50 µl	50 µl	50 µl	50 µl

* Skip this reaction if your RNA is nonhuman.
† Skip this reaction if your GSPs *will not* create overlapping RACE fragments.
For detailed descriptions of the control reactions, see Section XI.

4. Overlay the contents of each tube with 2 drops of mineral oil and place caps firmly on each tube.

   Note: Mineral oil is not necessary if you are using a hot-lid thermal cycler.

5. Commence thermal cycling using one of the following programs (both programs 1 and 2 work with the positive control 5'- and 3'-RACE TFR and UPM Primers). Be sure to choose the correct number of cycles (as noted) based on whether you started with poly A^+ or total RNA.

   Notes on cycling:
   Because the necessary number of cycles depends on the abundance of the transcript, you may need to determine the optimal cycling parameters for your gene empirically. Run 20 or 25 PCR cycles first as described and analyze 5 µl from each tube, along with appropriate DNA size markers, on a 1.2% agarose/EtBr gel. If you see weak bands or no bands, return the tube(s) to your PCR machine and perform five additional cycles (according to the third set of cycles for touchdown PCR). The optimal extension time depends on the length of the fragment being amplified. We typically use 3 min for cDNA fragments of 2–4 kb. For 0.2–2-kb targets, we reduce the extension time to 2 min. For 5–10-kb targets, we increase the extension time up to 10 min.

   Note: Figure 4 in Section VIII shows TFR sample results.

IX. Rapid Amplification of cDNA Ends (RACE) *continued*

Program 1 (preferred; use if GSP T_m >70°C)

- 5 cycles:

  94°C 30 sec
  72°C 3 min*

- 5 cycles:

  94°C 30 sec
  70°C 30 sec
  72°C 3 min*

- 20 cycles (Poly A^+ RNA):
  OR
- 25 cycles (Total RNA):

  94°C 30 sec
  68°C 30 sec
  72°C 3 min*

* If fragments >3 kb are expected, add 1 min for each additional 1 kb.

Program 2 (if GSP T_m = 60–70°C):

- 20 cycles (Poly A^+ RNA):
  OR
- 25 cycles (Total RNA):

  94°C 30 sec
  68°C 30 sec
  72°C 3 min*

* If fragments >3 kb are expected, add 1 min for each additional 1 kb.

6. [Optional] If the primary PCR reaction fails to give the distinct band(s) of interest or produces a smear, you may wish to perform a Southern blot using:
   a. A cDNA probe
   b. A nested primer as a probe

   Or, you may wish to perform a secondary, or "nested" PCR reaction using the NUP primer supplied and a NGSP. (See the discussion in Section V.)
   a. Dilute 5 µl of the primary PCR product into 245 µl of Tricine-EDTA buffer.
   b. Repeat Steps 1–5 above, using:
      - 5 µl of the diluted primary PCR product in place of the RACE-Ready cDNAs.
      - 1 µl of the NUP primer and 1 µl of your nested GSPs.
      - 15–20 cycles of Program 2.

X. Characterization of RACE Products

At this point, we recommend that you characterize your RACE fragments and confirm that you have amplified the desired product. This procedure can prevent confusion and wasted effort when you generate the full-length cDNA, even if you have single major products from both the 5'- and 3'-RACE reactions. Characterization is especially important if you have multiple bands or if you suspect that you are working with a member of a multigene family.

We describe three methods for characterizing RACE products: (A) Comparison of RACE products obtained with GSPs and NGSPs; (B) Southern blotting; and (C) Cloning and sequencing. Options A and B require nested GSPs for analyzing 5'- and 3'-RACE products. For more detailed blotting and cloning protocols, see Sambrook *et al*. (1989) or other appropriate laboratory manuals.

A. Comparison of RACE Products Obtained with GSPs & NGSPs

For the 5'-RACE reaction, compare the products of primary amplifications performed with the UPM Mix and GSP1 to the products obtained using the UPM and NGSP1. (For 3'-RACE, compare the products obtained from amplifications with the UPM and GSP2 to those obtained with the UPM and NGSP2.) This analysis will help determine if any multiple bands are a result of correctly primed PCR or nonspecifically primed PCR. If bands are real (i.e., the result of correct priming), they should be slightly smaller in the reaction using the nested gene-specific primers. The difference in mobility of the products should correspond to the positions of the outer and inner (nested) gene-specific primers in the cDNA structure. If you have multiple bands with UPM and GSP1 (or GSP2), some may disappear upon amplification with UPM and NGSP1 (or NGSP2).

Note: Do not use the Nested Universal Primer A (NUP) in these reactions, because it will cause a size decrease in *all* of the PCR products.

B. Southern Blot Analysis

You can obtain stronger confirmation of your RACE products by probing a Southern blot with an internal gene-specific probe (usually one of your other GSPs or NGSPs). This method can be particularly useful for determining which bands are real when RACE produces multiple bands. Multiple bands are more common with 5'-RACE than with 3'-RACE.

1. Examine your RACE products on an agarose/EtBr gel.
2. Photograph the gel, then transfer the DNA to a nylon membrane using standard blotting procedures.
3. Prepare a hybridization probe that does not have sequences in common with GSP1 (or GSP2). The probe can be end-labeled NGSP1 (or NGSP2). Alternatively, if your GSPs define overlapping 5' and 3' fragments, GSP2 can be used as a probe to characterize your 5'-RACE

X. Characterization of RACE Products *continued*

products, and GSP1 can be used as a probe to characterize your 3'-RACE products. Nick-translated or random-primed internal restriction fragments from a previously cloned partial cDNA can also be used.

4. Hybridize the probe to the Southern blot, wash under moderate-to-high stringency conditions, and expose x-ray film.
5. Compare the hybridization pattern to the photograph of the agarose/EtBr gel. Generally, you will want to isolate the RACE product(s) that correspond(s) to the largest band(s) on the Southern blot. There may be larger RACE products that appear on the agarose gel but that do not hybridize to the gene-specific probe. These bands are generally due to nonspecific priming. Smaller bands that hybridize to your probe may be the result of incomplete reverse transcription; however, you cannot exclude the possibility that some of these shorter bands are real and correspond to alternatively spliced transcripts, transcripts derived from multiple promoters, or other members of a multigene family.
6. Once you have pinpointed the band(s) of interest, isolate the DNA from the gel using the NucleoTrap Gel Extraction Kit provided, and proceed with your experiments.

C. Cloning & Sequencing RACE Products

Note: The Universal Primer contains a T7 priming site. Using a cloning vector that contains a T7 site will generate multiple sequencing products if using a T7 primer.

1. Gel-purify the RACE product(s) of interest using the NucleoTrap Gel Extraction Kit. Then, clone the isolated fragment(s) directly into a T/A-type PCR cloning vector.
2. After you have TA-cloned your RACE products, identify clones containing gene-specific inserts by colony hybridization using a ^{32}P-end-labeled NGSP as a probe or by sequencing from your GSP. For 5'-RACE products, we recommend picking at least 8–10 different independent clones in order to obtain the maximum amount of sequence at the 5' end.

   Once you have identified the clones containing the largest gene-specific inserts, obtain as much sequence data as you can. Ideally, you will be able to sequence the entire open reading frame, as well as the 5' and 3' untranslated regions.

X. Characterization of RACE Products *continued*

Options for generating full-length cDNA

After RACE products have been characterized by partial or complete sequencing, you can generate the full-length cDNA by one of two methods:

1. By long distance PCR (LD PCR) using primers designed from the extreme 5' and 3' ends of your cDNA and the 5'-RACE-Ready cDNA as template.
2. By cloning overlapping 5'- and 3'-RACE fragments using a restriction site in the overlapping region (if available).

In general, the LD PCR method is more direct and less subject to complications or artifacts. With cloning, it is possible to join 5' and 3' cDNA fragments derived from two different transcripts; this could occur with two different forms of a polymorphic RNA or with transcripts from a multigene family. In contrast, with end-to-end PCR, the 5' and 3' end primers will amplify a single cDNA, without the possibility of generating a hybrid. Virtually all cDNAs are within the range of LD PCR.

XI. Troubleshooting Guide

Optimizing your 5'- and 3'-RACE reactions is generally advisable and often necessary. This process usually consists of improving the yield of your desired fragment(s), while decreasing the amount of background or nonspecific and/or incomplete bands in your RACE reactions. The cDNA synthesis protocols contained in this User Manual typically produce enough 5'- and 3'-RACE-Ready cDNA for 100 or more RACE PCR reactions. Thus, there is plenty of material for optimizing your RACE amplifications.

A. Control PCR Reactions

Tables III and IV in the User Manual describe several control reactions that will help you troubleshoot the reactions if yields are suboptimal. These include:

- **Tube No. 2:** 5'- or 3'-RACE PCR using the positive control TFR Primer, the UPM Primer Mix, and the 5'- and 3'-RACE-Ready cDNA made from your experimental RNA. Figure 4 in Section VIII shows the expected results of 5'- and 3'- RACE using these positive controls.
- **Tube No. 3:** An additional positive control using both GSPs to amplify the overlapping segment of your 5'- and 3'-RACE fragments (if available). This reaction should give a single band corresponding to the overlap between the primers. This result confirms that your target cDNA is present in, and can be amplified from, your RACE-Ready cDNA. If you do not have suitable 5'- and 3'-GSPs (i.e., GSPs that create overlapping 5'- and 3'-RACE products), use the control 5'- and 3'-RACE TFR Primers with 5 µl of your positive control RACE-Ready cDNAs (if human).
- **Tube No.4:** A negative control using the UPM alone to amplify your cDNA. With fewer than 40 cycles, this reaction should produce no product. If this control produces a smear or ladder of extra bands, you may need to alter the cycling parameters or perform a secondary amplification using the Nested Universal Primer A.
- **Tube No.5:** A negative control using each GSP by itself. This control should produce no product. If this control produces a smear or ladder of extra bands, you may need to alter the cycling parameters, perform a secondary amplification using nested primers, or redesign your original primers.

B. General PCR Problems

- **Troubleshooting GC-rich templates:** If the PCR product, especially your 5'-RACE product, is not the expected size or is absent, the cause may be a GC-rich template. BD Biosciences Clontech offers the

XI. Troubleshooting Guide *continued*

BD Advantage GC 2 Polymerase Mix (Cat. No. 639114) and PCR Kit (Cat. Nos. 639119 & 639120) for efficient amplification of GC-rich templates. However, when using this polymerase mix or kit, the master mix recipes will need to be modified to include GC-Melt and the 5X PCR Reaction Buffer, instead of the 10X buffer supplied with most polymerases. Additionally, the PCR parameters may need to be optimized for these templates. For more information, please see the BD Advantage GC 2 PCR User Manual (PT3316-1). We recommend that you perform the initial RACE reactions with the BD Advantage 2 Polymerase Mix, then perform the RACE reactions using the BD Advantage GC 2 Polymerase Mix to confirm that the product is the same size in both reactions.

- **High-fidelity PCR:** If you are going to use your cloned RACE products for further analysis, we recommend that you generate your full-length cDNA using the BD Advantage HF 2 PCR Kit. (Cat. No. 639123) This kit is designed to yield products of less than 3.5 kb with fidelity comparable to the leading high-fidelity polymerase. This kit may not be ideal for cDNA templates that are greater than 3.5 kb, but it is especially well suited for applications in which the RACE product will be cloned for use in additional experiments. Again, the initial RACE reactions should be performed using the BD Advantage 2 Polymerase Mix to confirm that the product is present and that the GSPs work well.
- **Troubleshooting touchdown PCR:** When troubleshooting touchdown PCR, we recommend that you begin by modifying the final set of cycle parameters (i.e., the 20–25 cycles performed with annealing at 68°C). If you do not observe an amplified product after the minimum number of cycles at 68°C, return your tube(s) to the PCR machine and run five additional cycles. If the product still does not appear, add an additional 3–5 cycles at 68°C. If you are still unsuccessful, run a new PCR experiment, changing the annealing temperature in the third set of cycles from 68°C to 65°C. This last program is especially useful if your GSP has a T_m close to 70°C.

XI. Troubleshooting Guide *continued*

C. No band is observed in positive control amplification of the overlapping region of RACE products (either with GSP1 + GSP2 or TFR1 + TFR2).

The control PCR reaction using your sense and antisense GSPs and your RACE-Ready cDNA to amplify the internal fragment of your gene is very important. If this reaction fails to produce the expected internal cDNA fragment, there are at least two possible explanations:

- There may be a problem with your polymerase mix. If you are not using the BD Advantage 2 Polymerase Mix, consider switching. The BD SMART RACE protocol was optimized with the BD Advantage 2 Polymerase Mix. Be sure to perform the positive control PCR experiment in Section VIII.
- Your cDNA synthesis reaction may have failed. Repeat the first-strand synthesis reaction. You may wish to analyze the quality of your first-strand cDNA using the procedure described below in Section XI.K.

D. No band is observed using TFR1 + TFR2 with your experimental cDNA, but the correct product is seen with cDNA made from the positive control placental RNA.

Your RNA may be partially degraded or may contain impurities. Check the quality of your RNA against the criteria described in Section VI.

E. No band is observed using GSP1 + GSP2, but the correct product is seen using TFR1 + TFR2.

- This problem can be caused by the impeding of RT by strong secondary structure and/or high GC content in your gene. This is especially indicated if the 3'-RACE does work, the 5'-RACE does *not* work, and the positive control (GSP1 + GSP2) does *not* produce the expected fragment. See Section XI.B of the Troubleshooting Guide for help with GC-rich templates. Additionally you may wish to analyze the quality of your first-strand cDNA using the procedure described below in Section XI.K.
- Your gene may be expressed weakly or not at all in your starting RNA. You may have to find a new source of RNA. The efficiency of both 5'- and 3'-RACE amplifications depends on the abundance of the target transcript.
- There is a problem with your primers. This could be due either to poor primer design or poor primer preparation. First, try lowering your annealing/extension temperature. If this does not work, you may need to design new primers or repurify your GSPs.

You may be able to obtain more information by amplifying the internal fragment (with GSP1 and GSP2) using genomic DNA as the template. If the expected band is produced, your primers are suitable and the problem is either (a) the target RNA is a poor template for RT; or (b) the RNA is not

XI. Troubleshooting Guide *continued*

expressed in the tissue source you have chosen. Note, however, that this test is not conclusive, since your primers may be separated by an intron in the genomic DNA. If this is the case, amplification of genomic DNA will give a larger fragment than expected or no fragment at all.

F. The 3'-RACE works, but the 5'-RACE does not in both experimental and TFR amplification.

- This is often the result of a failure in full-length cDNA synthesis and/or the template switching reaction. Try repeating the first-strand synthesis reaction. You may wish to analyze the quality of your first-strand cDNA using the procedure described below in Section XI.K.
- Your RT may be degraded. This can happen if BD PowerScript RT is not kept on ice at all times, or if it is not returned to the freezer promptly after use.

G. No bands are observed in any RACE reactions using either gene-specific or positive control primers with either experimental or control RNA samples.

If you still do not observe RACE products after 25–30 cycles of PCR (especially in *both* 5'- and 3'-RACE reactions), return the tubes to your PCR machine and perform 5 additional cycles. You may have to optimize the PCR program for your thermal cycler. If these steps do not resolve the problem, it may be that the cDNA synthesis and/or template switching reaction has failed. In this case try repeating the cDNA synthesis reactions. You may wish to analyze the quality of your first-strand cDNA using the procedure described below in Section XI.K.

H. Using your experimental cDNA sample, no 5'- or 3'-RACE bands are produced, but the TFR positive control RACE reactions (Tube No. 2) give the expected products.

- Your gene may not be abundant in your RNA sample. Perform 5 more PCR cycles at the 68°C annealing temperature. Repeat these additional cycles until your RACE fragments appear, but do not exceed 50 cycles for touchdown PCR or 40 cycles for non-touchdown PCR. If you still fail to produce the expected products, you may have to find a new source of RNA in which your gene is more abundant.
- The annealing temperature is too high for your primers. Lower the annealing temperature by increments of 2°C.
- Your primers are not suitable for PCR. Check them against the criteria in Section V, and design new ones if necessary.
- The structure of the gene is difficult for PCR due to secondary conformations or high GC-content. Try redesigning your primers closer to the ends of the cDNA, or try to avoid GC-rich regions if they are known. For additional tips in troubleshooting GC-rich sequences, see Section XI.B of the Troubleshooting Guide.

XI. Troubleshooting Guide *continued*

- Your gene is too long for RT and/or LD PCR. Design your primers as close to the ends as possible. Then repeat the 5'-RACE-Ready cDNA synthesis using either a GSP or random hexamers to prime reverse transcription instead of the 5'-CDS Primer provided.

I. RACE product consists of multiple bands.

In some cases, your initial experiments will produce multiple 5'- and/or 3'-RACE products. As mentioned above, you will have to determine which products are real and which are artifacts. While the following guidelines will help you eliminate artifacts, confirmation of real and complete bands requires additional studies such as mapping of transcription start sites, intron/exon structure and polyadenylation sites, and genomic sequencing.

Multiple fragments do not mean you cannot proceed with generating the full-length cDNA. However, you may save time in the long run if you try to eliminate nonspecific fragments by troubleshooting the reactions. If multiple fragments persist and you want to proceed, you should generally start with the largest fragment from each RACE reaction, because it is most likely to be a true, complete RACE product.

Sources of "real" multiple RACE products

Individual genes can give rise to multiple sizes of transcripts—and hence to multiple RACE fragments—via at least three mechanisms:

- Alternative splicing can cause multiple products in 5'- or 3'-RACE.
- Use of different transcription initiation sites causes multiple 5'-RACE products.
- Use of different polyadenylation sites causes multiple 3'-RACE products.

Alternatively, the gene may be a member of a multigene family, in which case your "gene-specific" primers may simultaneously amplify several highly homologous cDNAs.

Distinguishing true polymorphic forms of an RNA is a matter for scientific investigation. However, you may be able to find an alternative source of RNA in which one form is more abundant than others.

XI. Troubleshooting Guide *continued*

Sources of artifacts

Multiple bands often do not correspond to actual, complete transcripts. These artifact RACE products can be divided into two classes—incomplete and nonspecific.

There are several possible sources of incomplete fragments, which are generated from correctly primed sites.

- Premature termination of first-strand cDNA synthesis caused by RT pausing generally causes multiple 5'-RACE products. This problem is common with larger RNAs, and is difficult to overcome, because it is due to an intrinsic limitation of RT.
- Degradation of the RNA used as starting material generally causes multiple 5'-RACE products.
- Difficulty in amplifying certain genes can cause multiple products in either 5'- or 3'-RACE and is often a result of high GC content.

Nonspecific RACE products arise from nonspecific binding of the primer to multiple sites in the ds cDNA or primer-dimer artifacts.

Suggestions:

- If you have not already done so, repeat your RACE reactions with all of the recommended controls. In particular, be sure that your GSPs do not give bands when used alone, and that they give a single band when used together. If either GSP alone gives persistent bands, we recommend altering the cycling parameters or designing nested primers as discussed below. Also repeat the Positive Control RACE PCR Experiment.
- Repeat your reactions using 5 µl of a 5–10-fold lower dilution of the RACE-Ready cDNA.
- If you have not already done so, examine the size distribution of your RNA starting material as discussed in Section VI. If your RNA looks smaller than expected, repurify your RNA and repeat cDNA synthesis.
- If multiple bands persist, try altering the PCR cycling parameters:
    1. Increase the stringency of your PCR by raising the annealing temperature in increments of 2–5°C. In many cases, bands arising from nonspecific priming will disappear while real or incomplete products will persist.
    2. Reduce the cycle number. Again, bands arising from nonspecific priming may disappear, while real or incomplete products will persist.
    3. Reduce the extension time.
    4. In the case of large RACE products, increasing the extension time may help eliminate extra bands.

XI. Troubleshooting Guide *continued*

- If multiple bands persist, try designing a new set of primers:
  1. Redesign your primers so that they have a T_m greater than 70°C and use the cycling parameters for touchdown PCR.
  2. We recommend that you design new primers that will give RACE products that are slightly different in size than those expected with the original primers. These new primers can either be used by themselves or in combination with the original primers in "nested PCR". In nested PCR the product of a PCR reaction is reamplified using a second set of primers that is internal to the original primers. This often greatly reduces the background and nonspecific amplification seen with either set of primers alone. The design of nested primers is discussed in Section V.
  3. Prior to performing nested RACE PCR, we recommend that you perform two separate primary amplifications with the UPM and either the GSP1 or NGSP1. This test will help show if multiple bands are a result of correctly primed PCR or nonspecifically primed PCR. If the multiple bands are real (i.e., the result of correct priming), they should be present in both reactions, but slightly smaller in the reaction using the nested primers. The difference in mobility of the products should correspond to the positions of the GSP and NGSP in the cDNA structure.

If none of the above suggestions works, you may want to try repeating cDNA synthesis using a GSP or random hexamers instead of the CDS Primer provided with this kit.

J. RACE cDNA product is smeared.

Note: Some BD SMART RACE reactions produce very complex patterns of bands that appear almost as smears.

In most cases of true smearing, a problem has occurred prior to the RACE reaction, *especially if the 3'-RACE reaction produces a smear*. Smearing of *only* the 5'-RACE reaction products may indicate a difficult template for reverse transcription or degraded RNA. Smearing of *both* reactions is a strong indication of contamination of your starting RNA or a problem in reverse transcription. In these cases we recommend repeating the entire procedure after repurifying your RNA (or confirming that your RNA is intact and clean). See Section VI for more details.

If smearing is apparently not due to a problem that occurred prior to RACE, try optimizing your RACE reactions using the troubleshooting tips described above for multiple bands.

XI. Troubleshooting Guide *continued*

K. Analyzing the quality of first-strand cDNA.

If you suspect that problems amplifying your RACE fragments may be due to a failure of the reverse transcription reaction, you can check the quality of first-strand cDNA (if generated from poly A^+ RNA) using a ^{32}P-labeling procedure. To do this, repeat the first-strand synthesis, substituting 1µl of 0.1 µCi/µl [α-^{32}P] dATP or dCTP for one of the microliters of water. Run the reaction products on an alkaline agarose gel, and examine the banding pattern by autoradiography. If the first-strand reaction was successful, you should see a similar banding pattern to that produced by your RNA. Mammalian poly A^+ RNA typically produces a smear from 0.5–12 kb. Mammalian total RNA usually exhibits two bright bands at 4.5 and 1.9 kb.

XII. References

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XIII. Related Products

For a complete listing of all BD Biosciences Clontech products, please visit **www.bdbiosciences.com/clontech**

	Cat. No.	New Cat. No.
• BD™ Marathon-Ready cDNAs	many	many
• BD Clontech™ PCR-Select cDNA Subtraction Kit	K1804-1	637401
• BD Delta™ Differential Display Kit	K1810-1	637405
• BD Advantage™ 2 PCR Kit	K1910-1 K1910-y	639206 639207
• BD Advantage™ 2 Polymerase Mix	8430-1 8430-2	639201 639202
• BD Sprint™ Advantage™ Single Shots	K1953-1 K1953-2 K1953-y	639553 639554 639556
• BD Sprint™ Advantage™ 96 Plate	K1950-1	639550
• BD Sprint™ Combo Kit	K1951-1	639551
• BD Advantage™ GC 2 PCR Kit	K1913-1 K1913-y	639119 639120
• BD Advantage™ GC 2 Polymerase Mix	8433-1	639114
• BD Advantage™ HF 2 PCR Kit	K1914-1 K1914-y	639123 639124
• NucleoBond® RNA/DNA Mini Kit	K3025-1	635945
• NucleoTrap® Gel Extraction Kit	K3070-1	636018
• NucleoTrap® PCR Purification Kit	K3071-1	636020

Appendix A: Detailed Flow Chart of 5'-RACE

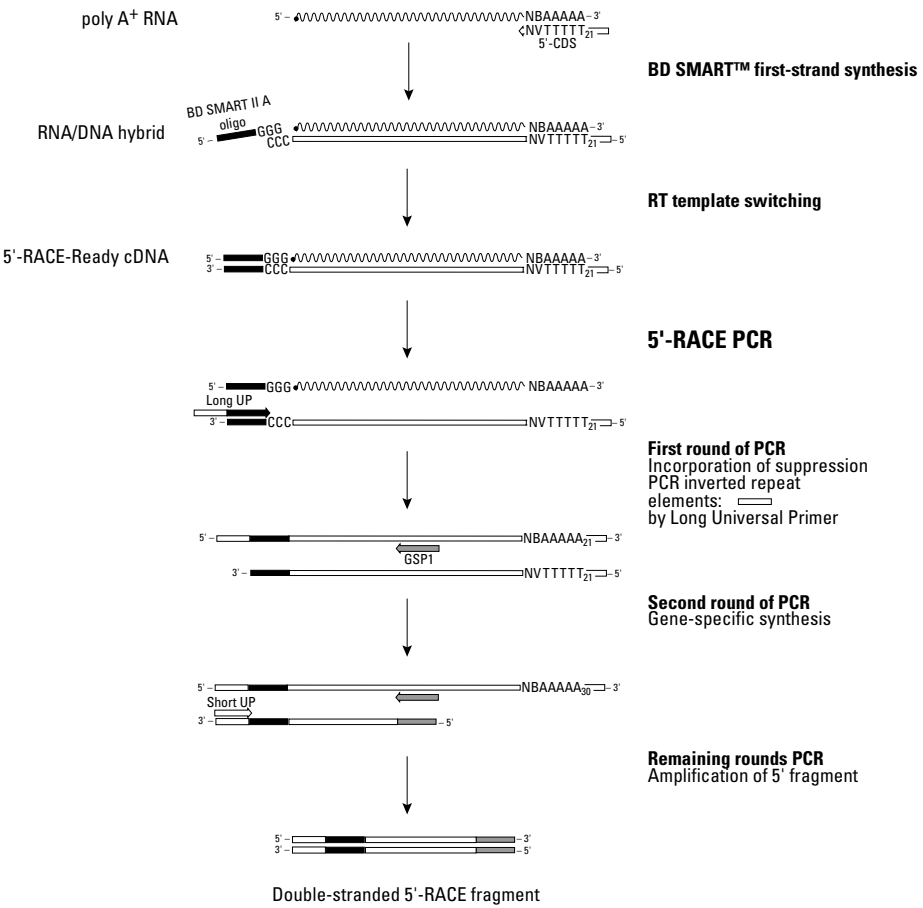

Figure 5. Detailed mechanism of the 5'-RACE reactions.

Appendix B: Detailed Flow Chart of 3'-RACE

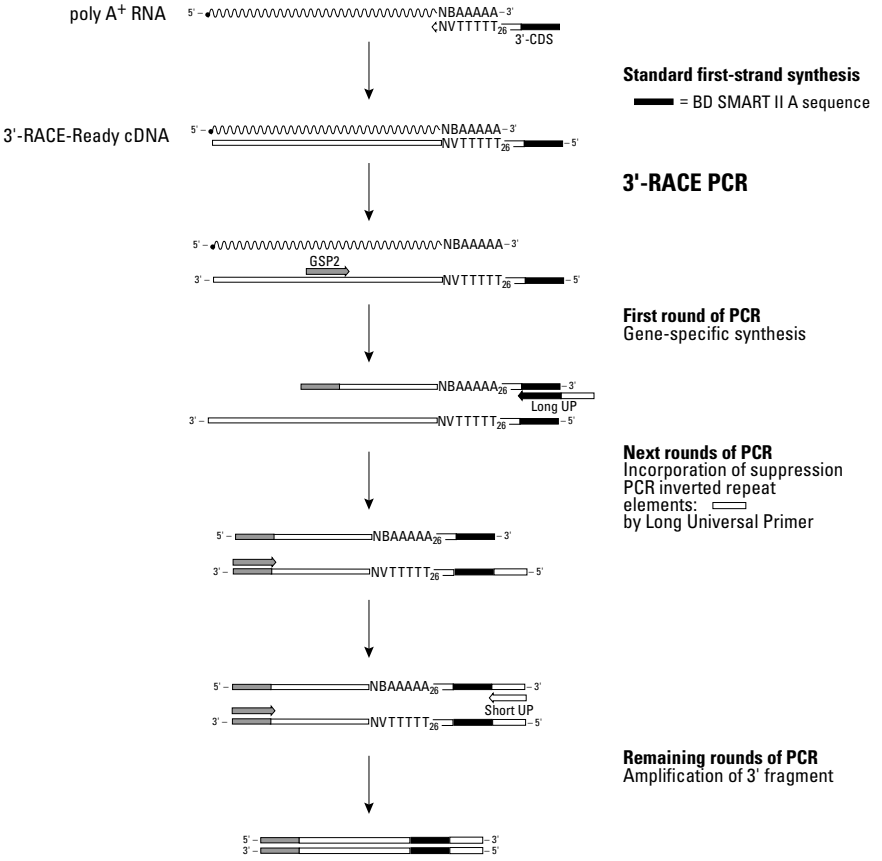

Figure 6. Detailed mechanism of the 3'-RACE reactions.

Appendix C: Suppression PCR and Step-Out PCR

In our initial BD SMART-based 5'-RACE experiments, we tended to observe a heavy background amplification. We determined that undesired bands were produced by nonspecific priming of reverse transcriptase by the BD SMART II A oligo during synthesis of the 5'-RACE-Ready cDNA. When reverse transcription and template switching are completed, these cDNAs contain the BD SMART sequence at *both* ends (Figure 7). As a result, RACE PCR using primers based on the BD SMART sequence amplifies these cDNAs in addition to the desired gene specific fragment. This problem was overcome with the use of suppression PCR and step-out PCR.

In suppression PCR (Siebert *et al.*, 1995), an inverted repeat is incorporated in the ends of DNA sequences to prevent amplification during PCR. The suppression effect occurs when these inverted repeats anneal intramolecularly to form panhandle structures which cannot be amplified by PCR (see the bottom of the flow chart in Figure 7). The BD SMART RACE Kit uses the technique of step-out PCR to add these inverted repeats and thus suppress the amplification of cDNA species that were synthesized by BD SMART II A oligo priming during reverse transcription. Step-out PCR uses a mixture of two primers to incorporate additional sequence at the end/s of template DNA (Matz *et al.*, 1999). One of these primers is exceptionally long and contains the additional sequence as a non-annealing overhang. The overhang sequence is incorporated into template DNA ends in the early rounds of PCR. After overhang addition, the second primer, which is only complementary to the overhang sequence, takes over and serves as an efficient primer for PCR amplification. This short primer is essential because the bulky incorporation primer is inadequate for effective amplification. The short primer is included at a higher concentration than the long primer so that it out-competes the long primer in annealing to template DNA during PCR.

In this same manner, the Universal Primer A Mix adds suppression PCR inverted repeat elements to ends of cDNAs in BD SMART RACE. One of the primers in the mix, the “Long” Universal Primer (UP), is complementary to the BD SMART sequence at its 3' end and also has a 5' heel of 20 bp which contains the suppression sequence (Figure 7). During the early rounds of RACE PCR, this primer incorporates the suppression sequence on the 5' side of all BD SMART sequences present in the cDNA population. As a result, all cDNAs that were correctly primed by oligo(dT) and only have one BD SMART sequence at the 3' end of the first-strand cDNA will contain one suppression sequence at that end. Conversely, all cDNAs that were primed by the BD SMART II A oligo, and which were consequently flanked by the BD SMART sequence, become flanked again by the inverted repeat and are subject to suppression PCR. Therefore, cDNAs that have the BD SMART sequence on only one end *and* the gene specific sequence will be amplified exclusively. As described above, the “Short” UP, which is present at five times the concentration of the Long UP, only contains the 5'-heel sequence of the Long UP, and simply serves as an efficient PCR primer *after* incorporation of the inverted repeat.

Appendix C: Suppression PCR and Step-Out PCR *continued*

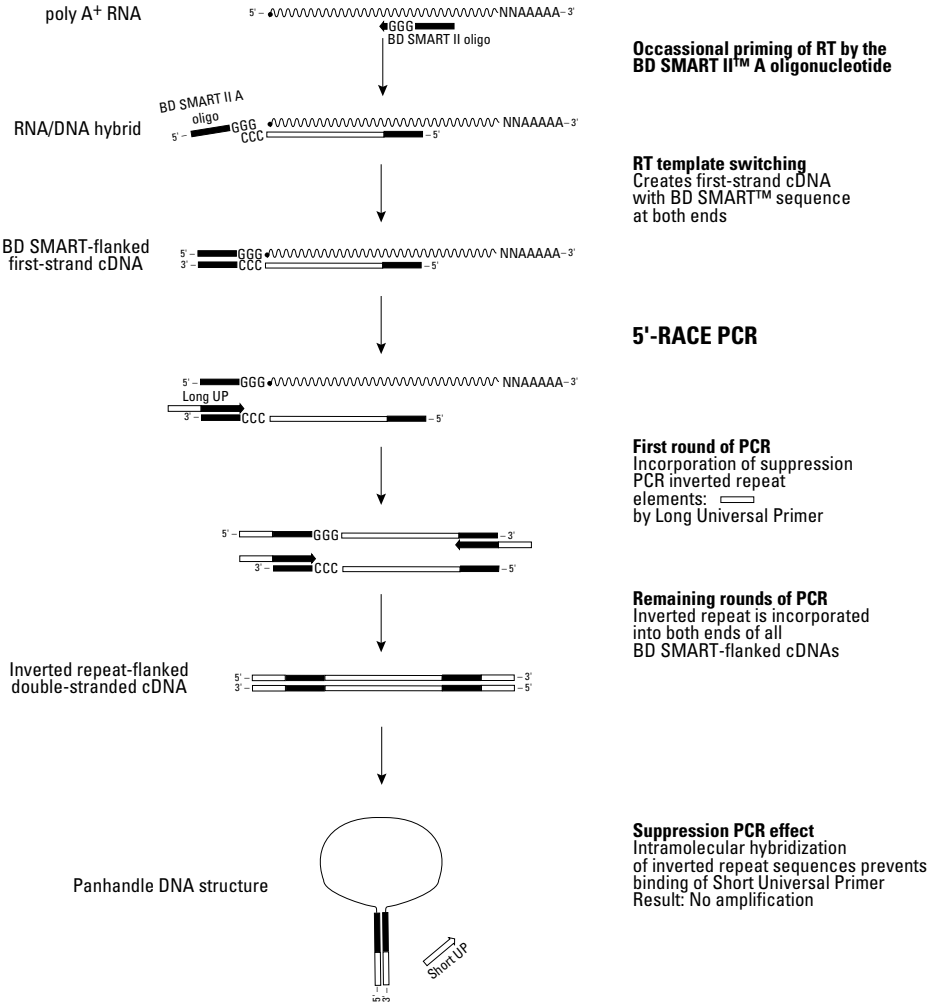
poly A+ RNA
5' – NNAAAAA– 3'
GGG
BD SMART II oligo
Occassional priming of RT by the BD SMART II™ A oligonucleotide
BD SMART II A oligo
RNA/DNA hybrid
GGG
CCC
RT template switching
Creates first-strand cDNA with BD SMART™ sequence at both ends
BD SMART-flanked first-strand cDNA
5'-RACE PCR
Long UP
First round of PCR
Incorporation of suppression PCR inverted repeat elements: by Long Universal Primer
Remaining rounds of PCR
Inverted repeat is incorporated into both ends of all BD SMART-flanked cDNAs
Inverted repeat-flanked double-stranded cDNA
Panhandle DNA structure
Short UP
Suppression PCR effect
Intramolecular hybridization of inverted repeat sequences prevents binding of Short Universal Primer
Result: No amplification

Figure 7. Mechanisms of suppression PCR and step-out PCR. On occasion, a reverse transcription reaction can be "nonspecifically" primed by the BD SMART II A oligonucleotide. This will result in the synthesis of a cDNA containing the BD SMART sequence at both ends. Through the technique of step-out PCR, suppression PCR inverted repeat elements are incorporated next to all BD SMART sequences. During PCR, these inverted repeats anneal to each other intramolecularly. This rapid first-order reaction out-competes the second-order binding of the Short Universal Primer to the cDNA. As a result, panhandle-like structures, which cannot be amplified, are formed.

Notes

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